Corporation obtaining approval, the name of its representative, and the address of its main office

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Approved Type 1 Use Regulation

Name of the Type of Living Modified Organism	Glufosinate herbicide tolerant, male sterile and fertility restored oilseed rape (Modified *bar, barnase, barstar, Brassica napus* L.) (MS1RF2, OECD UI :ACS-BNØØ4-7×ACS-BNØØ2-5)
Content of the Type 1 Use of Living Modified Organism	Provision as food, provision as feed, processing, storage, transportation, disposal and acts incidental to them
Method of the Type 1 Use of Living Modified Organism	—

Outline of the Biological Diversity Risk Assessment Report

I. Information collected prior to assessing Adverse Effect on Biological Diversity

1. Information concerning preparation of living modified organisms

(1) Information concerning donor nucleic acid

1) Composition and origins of component elements

Glufosinate herbicide tolerant and male sterile and fertility restored oilseed rape (modified *bar, barnase, barstar, Brassica napus* L., MS1RF2, OECD UI: ACS-BNØØ4-7 x ACS-BNØØ 2-5) (hereinafter referred to as "MS1RF2") is a hybrid obtained by a cross between glufosinate herbicide tolerant and male sterile oilseed rape (modified *bar*, *barnase*, *Brassica napus* L., MS1, OECD UI: ACS-BNØØ4-7) (hereinafter referred to as "MS1") and glufosinate herbicide tolerant and fertility restored oilseed rape (modified *bar*, *barstar*, *Brassica napus* L., RF2, OECD UI: ACS-BNØØ2-5) (hereinafter referred to as "RF2"). Composition of the donor nucleic acid that was used for the production of MS1 and RF2, the parent plants of MS1RF2, and the origins of component elements are shown in Table 1-1 and Table 1-2 respectively.

For the wild-type *bar* gene obtained from *Streptomyces hygroscopicus*, GTG was modified to ATG to conform to frequently-used codons in plant, and AGC was modified to GAC to improve efficiency of translation. Regarding the translated amino acid, methionine remains unchanged in the modification from GTG to ATG, though serine changes to asparatic acid in the modification from AGC to GAC. However, it is confirmed that the function of modified PAT protein produced by the modified *bar* gene (hereinafter referred to as "the modified PAT protein") remains unchanged in this modification (Reference 98).

The nucleotide sequences of modified *bar* gene, *barnase* gene and *barstar* gene are shown in Figures 1-1, 1-2 and 1-3 respectively.

Table 1-1 Origin and function of component elements (MS1)

Component elements	Size (kbp)	Origin and function
Modified *bar* gene expression cassette		
3'g7	0.2	3' untranslated region of nopaline synthase gene derived from pTiB6S3. It terminates transcription and causes 3' polyadenylation (References 21 and 96).
Modified *bar*	0.6	A gene encoding phosphinothricin acetyl transferase (modified PAT protein) derived from *Streptomyces hygroscopicus* and conferring tolerance to glufosinate herbicide (Reference 93). The two codons, GTG and AGC, in the N-terminal of wild-type *bar* gene are replaced for ATG and GAC respectively.
PSsuAra	1.9	Composed of the promoter SsuAra of RuBisCo small subunit gene which is derived from *Arabidopsis thaliana* and induces expression selectively in the chlorenchyma (Reference 47) and the transit peptide (TP) sequence targeted at the chloroplast.
barnase gene expression cassette		
3'nos	0.3	3' untranslated region of nopaline synthase gene derived from pTiT37. It terminates transcription and causes 3' polyadenylation (Reference 20).
barnase	0.3	Derived from *Bacillus amyloliquefaciens*. It expresses RNA-degrading enzyme (ribo-nuclease) in the tapetum cell to confer male sterility (Reference 30).
PTA29	1.5	A promoter of anther-specific gene TA29 derived from *Nicotiana tabacum*. It induces specific expression in the tapetum cell (Reference 80).
neo gene expression cassette		
Pnos	0.4	A promoter of nopaline synthase gene derived from pTiT37 of *Agrobacterium tumefaciens*. It initiates transcription of *neo* gene in plants (Reference 20).
Neo	1.0	Derived from transposon Tn5 of *Escherichia coli*, encoding neomycin phosphotransferase II (NPT II) and conferring resistance to aminoglycoside derivative antibiotics (Reference 4). The ATG initiation codon has been replaced for linker sequence (Reference 72).
3'ocs	0.9	3'-terminal regulated region of octopine synthase gene derived from *Agrobacterium tumefaciens*, terminating transcription and inducing polyadenylation of transcripts (Reference 19).
Additional information		
RB	0.02	It is the right border of the T-DNA derived from pTiB6S3.
LB	0.02	It is the left border of the T-DNA derived from pTiB6S3.

(Note: All the rights pertinent to the information in the table above and the responsibility for the contents rest upon the applicant.)

Table 1-2 Origin and function of component elements (RF2)

Component elements	Size (kbp)	Origin and function
Modified *bar* gene expression cassette		
3'g7	0.2	3' untranslated region of nopaline synthase gene derived from pTiB6S3. It terminates transcription and causes 3' polyadenylation (References 21and 96).
Modified *bar*	0.6	A gene encoding phosphinothricin acetyl transferase (modified PAT protein) derived from *Streptomyces hygroscopicus* and conferring tolerance to glufosinate herbicide (Reference 93). The two codons, GTG and AGC, in the N-terminal of wild-type *bar* gene are replaced for ATG and GAC respectively.
PSsuAra	1.9	Composed of the promoter SsuAra of RuBisCo small subunit gene which is derived from *Arabidopsis thaliana* and induces expression selectively in the chlorenchyma (Reference 47) and the transport peptide (TP) sequence targeted at the chloroplast.
barstar gene expression cassette		
3'nos	0.3	3' untranslated region of nopaline synthase gene derived from pTiT37. It terminates transcription and causes 3' polyadenylation (Reference 20).
barstar	0.3	It encodes ribo-nuclease inhibitor (BARSTAR protein), derived from *Bacillus amyloliquefaciens*. BARSTAR protein binds to ribo-nuclease, the product of *barnase* gene specifically, and inhibits its activity to restore male sterility (Reference 30).
PTA29	1.5	A promoter of anther-specific gene TA29 derived from *Nicotiana tabacum*. It induces specific expression in the tapetum cell (Reference 80).
neo gene expression cassette		
Pnos	0.4	A promoter of nopaline synthase gene derived from pTiT37 of *Agrobacterium tumefaciens*. It initiates transcription of *neo* gene in plants (Reference 20).
Neo	1.0	Derived from transposon Tn5 of *Escherichia coli*, encoding neomycin phosphotransferase II (NPT II) and conferring resistance to aminoglycoside derivative antibiotics (Reference 4). The ATG initiation codon has been replaced for linker sequence (Reference 72).
3'ocs	0.9	3'-terminal regulated region of octopine synthase gene derived from *Agrobacterium tumefaciens*. It terminates transcription and induces polyadenylation of transcripts (Reference 19).
Additional information		
RB	0.02	It is the right border of the T-DNA derived from pTiB6S3.
LB	0.02	It is the left border of the T-DNA derived from pTiB6S3.

(Note: All the rights pertinent to the information in the table above and the responsibility for the contents rest upon the applicant.)

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Figure 1-1 Nucleotide sequence of the modified *bar* gene

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Figure 1-2 Nucleotide sequence of the *barnase* gene

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Figure 1-3 Nucleotide sequence of the *barstar* gene

2) Functions of component elements

(a) Functions of target genes, expression-regulating regions, localization signals, selectable markers and other component elements of donor nucleic acid

Functions of component elements of donor nucleic acid which were used for the production of MS1 and RF2 are shown in Table 1-1 and Table 1-2 respectively.

(b) Functions of proteins produced by the expression of target genes and selective markers, and the fact, if applicable, that the produced protein is homologous with any protein which is known to possess any allergenicity

[Modified PAT protein]

In the process of nitrogen metabolism, crops produce ammonia by nitrate reduction, amino acid degradation, photorespiration, and so on. Glutamine synthetase plays an important role in detoxification of the ammonia produced, though the glutamine synthetase is inhibited if crops are sprayed with glufosinate herbicide, ammonia accumulates, and the crops wither and die.

Phosphinothricin acetyl transferase (modified PAT protein) produced by the transferred modified *bar* gene acetylates the glufosinate to make N-acetylglufosinate, which inactivates the inhibition of glutamine synthetase by the glufosinate. Then ammonia is not accumulated in the plant body, and the crop does not die even if it is sprayed with glufosinate herbicide.

The modified PAT protein exhibits a high affinity to glufosinate. Glufosinate is classified into L-amino acid, though it does not cause any acetyl group transfer reaction to the other various amino acids and it has little affinity for the glutamic acid which has specifically high structural similarity to glufosinate

and it causes virtually no transfer reaction *in vivo* (Reference 93). In addition, even in the presence of excessive amount of various amino acids, the acetyl group transfer reaction to glufosinate by modified PAT protein was never inhibited (Reference 98). As a result, it is considered that the modified PAT protein possesses high substrate specificity to glufosinate.

[BARNASE protein]

BARNASE protein is a single stranded protein consisting of 110 amino acids, and it degrades RNA by two-stage reaction mode. It breaks the 3',5'-phosphodiester bond of the polyribonucleotide strand, transfers the phosphate group to 2'-OH group of the ribose and produces the 2',3'-cyclic nucleotide as an intermediate (the first-stage phosphotransfer reaction). Then, the BARNASE protein hydrolyzes the intermediate and produces the 3'-nucleotide specifically (the second-stage hydrolysis reaction) (Reference 33). It possesses high specificity for breaking the 3'-site of guanine, but it also breaks the other site, therefore only mono-nucleotide and di-nucleotide are detected from the complete degradation products (Reference 76).

Pollens are produced during the highly controlled process in an anther. Tapetum cell, one of the tissues of the anther, plays an important role such as providing nutrition at the time of pollen formation and during the growth of pollens. Therefore, it is considered that the absence of tapetum cells in the pollen production is the major factor of resulting male sterility (Reference 43).

The *barnase* gene expresses the ribo-nuclease (BARNASE protein) which hydrolyzes the single stranded RNA molecule in the tapetum cell layer of the anther under the control of the promoter PTA29. The BARNASE protein degrades the RNA in the tapetum cells and thus the plants are unable to produce pollens (References 23, 31 and 50). It is also shown that the *barnase* gene, under the control of the promoter PTA29, expresses stably even in the high-temperature condition (37℃ during the daytime) (Reference 2). It is not reported that the promoter PTA29 induces temperature-dependent expression.

[BARSTAR protein]

BARSTAR protein is an intracellular inhibitor for BARNASE protein (References 28 and 31). BARSTAR protein forms non-covalently bonded complex specifically with BARNASE protein in one-to-one correspondence and inhibits the ribo-nuclease activity of BARNASE protein completely (References 29, 31 and 85).

In general, the first cross cultivar (F1 cultivar) possesses stronger and higher productivity, and excellent uniformity compared to the fixed cultivar (Reference 46). However, it is hard to obtain the F1 cultivar without fail for self-fertile crops such as oilseed rape. It becomes possible to obtain the F1 seeds without fail by crossing the female strain (the recombinant oilseed rape MS1 to which *barnase* gene was transferred to express specifically in the tapetum cell of the anther and inhibit the production of pollens) with the male

strain (the recombinant oilseed rape RF2 which possesses the trait to restore pollen fertility). In the F1 generation, pollen fertility is restored by the function of BARSTAR protein which inhibits the BARNASE protein (Reference 51), therefore, the seed production in high-yield by self-pollination becomes available.

[Toxicity and allergenicity of modified PAT protein, BARNASE protein and BARSTAR protein]

For the amino acid sequence of modified PAT protein, BARNASE protein and BARSTAR protein, homology search with known allergens in the database (Swiss Prot, PIR and HIV-AA) was conducted. In addition, shorter allergen epitope search was also conducted. Consequently, in the both searches, no homology with known toxins and allergens was observed.

(c) Contents of any change caused to the metabolic system of recipient organism

[Modified PAT protein]
Since the modified PAT protein possesses high substrate specificity (Reference 93), it is considered that it does not cause any acetyl group transfer reaction to the compounds other than glufosinate. Therefore, it is considered that the modified PAT protein does not affect the metabolic pathway of the recipient organism.

[BARNASE protein]
The expression of the *barnase* gene is limited to the tapetum cells under the control of the promoter PTA29 (Reference 50), and the *barnase* gene is unlikely to be expressed in any other tissue. The tapetum cell mostly develops at the 4-tetrad stage of pollen production, and degrades/breaks along with the development of pollens (Reference 88). Therefore, it is considered that the possibility of the *barnase* gene to express in the tissues other than tapetum cells and to affect the metabolic pathway of the plant body is extremely low.

[BARSTAR protein]

The *barstar* gene is under the control of the promoter PTA29 and then it is unlikely to be expressed in any tissue other than tapetum cell. In addition, BARSTAR protein forms non-covalently bonded complex specifically with BARNASE protein in one-to-one correspondence, and the stability of the complex is high (References 49 and 52). Furthermore, the ribo-nuclease of bacteria and filamentous fungi is found considerably homologous in the structure and sequence and thus, it is expected that these enzymes contain some inhibitors homologous with BARNASE protein. However, such inhibitors are known only in the ribo-nuclease BINASE protein produced by the *Bacillus intermedius*. The BINASE protein possesses high homology (85%) with the BARNASE protein, and it is inhibited by the BARSTAR protein (Reference 101). Its homology with the amino acid sequence of BARNASE protein is only 20 to 25%, though there is a report that the extra-cellular ribo-nuclease of *Streptomyces*, which has the similar protein structure, (Reference 35), is also inhibited by the BARSTAR protein (Reference 32). However, it is not reported that the BARSTAR protein exhibits the inhibiting activity against the ribo-nuclease in plants. The BARSTAR protein is reported not

to bind to any ribo-nuclease of human or animals (References 30, 31, 35 and 85). Based on the above understanding, it is considered that BARSTAR protein would not affect the metabolic system of the recipient organism.

(2) Information concerning vector

1) Name and origin

The vector used for the production of MS1 and RF2 is pTTM8RE and pTVE74RE respectively, which were both constructed based on the vector pGV825 (Reference 17).

2) Properties

(a) The numbers of base pairs and nucleotide sequence of vector

The total number of base pairs of the vectors pTTM8RE and pTVE74RE is 15,339bp and 15,225bp respectively. The entire nucleotide sequences of the both vectors are shown in Annex 1-1 and 1-2. In addition, the physical map of vector and the restriction enzyme cleavage site are shown in Figure 2 and Figure 3 respectively.

(b) Presence or absence of nucleotide sequence having specific functions, and the functions

The plasmids pTTM8RE and pTVE74RE possess the streptomycin/spectinomycin tolerance gene (*Sm/Sp*), kanamycin tolerance gene (*KanR*), ORIpBR, and *barstar* gene outside the T-DNA region. The *Sm/Sp* and *KanR* were used as selectable markers for the vectors. In addition, ORIpBR is the replication origin, which functions to cause autonomous replication in the *E.coli*. Furthermore, the *barstar* gene has been present in the basic plasmid used for constructing the both plasmids. At the time of inserting the *barnase* gene to the plasmid by using *E.coli* for constructing the pTTM8RE, a small amount of BARNASE protein would express even though using the promoter for plants and the *E.coli* would die. Therefore, the *barstar* gene was used for inhibiting the enzyme activity of the BARNASE protein. These sequences locate outside the T-DNA region, and they are considered not to be transferred into the oilseed rape genomes (Annex 2).

(c) Presence or absence of infectious characteristics of vector and the information concerning the region of recipient organism if the infectivity of vector is found present

It is known that the range of recipient organisms for the autonomous replication of plasmids pTTM8RE and pTVE74RE is limited to *Agrobacterium tumefaciens*, *E.coli* and gram-negative bacteria, and the plasmids do not possess the infectious characteristics in plants.

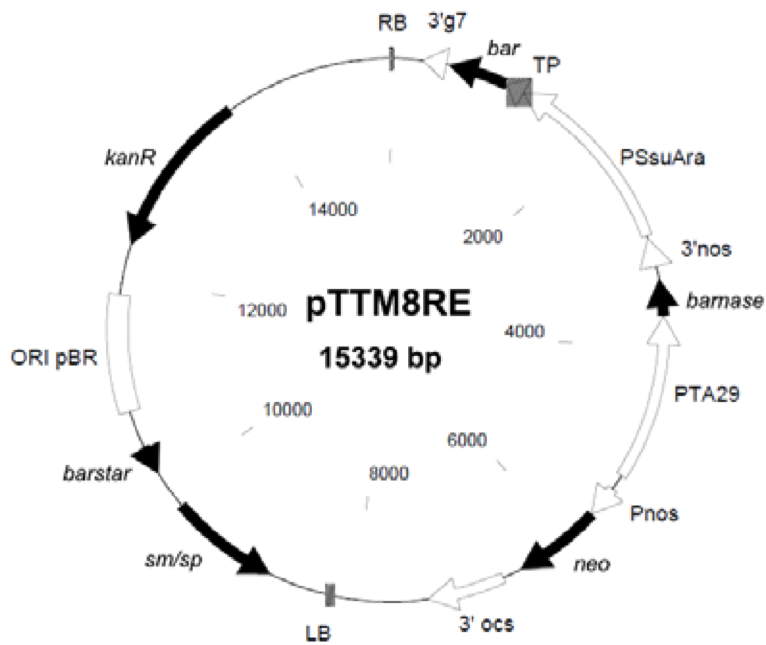

Figure 2-1 Physical map of plasmid pTTM8RE

(Note: All the rights pertinent to the information in the diagram above and the responsibility for the contents rest upon the applicant.)

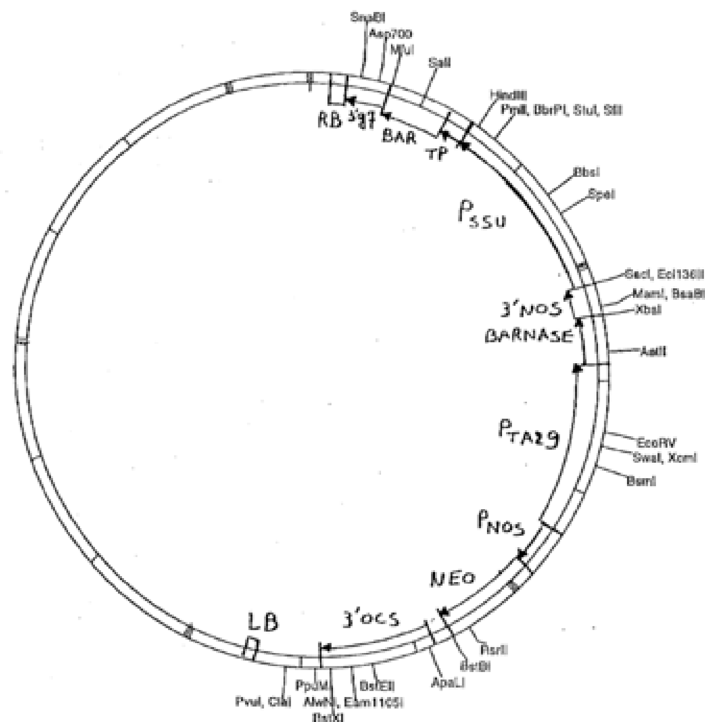

Figure 2-2 The restriction enzyme cleavage site of pTTM8RE

(Note: All the rights pertinent to the information in the diagram above and the responsibility for the contents rest upon the applicant.)

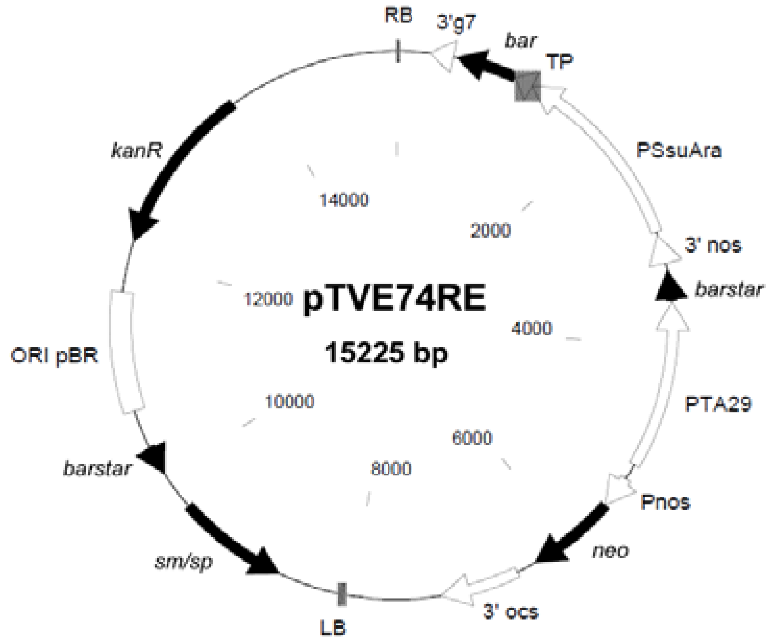

Figure 3-1 Physical Map of plasmid pTVE74RE

(Note: All the rights pertinent to the information in the diagram above and the responsibility for the content rest upon the applicant.)

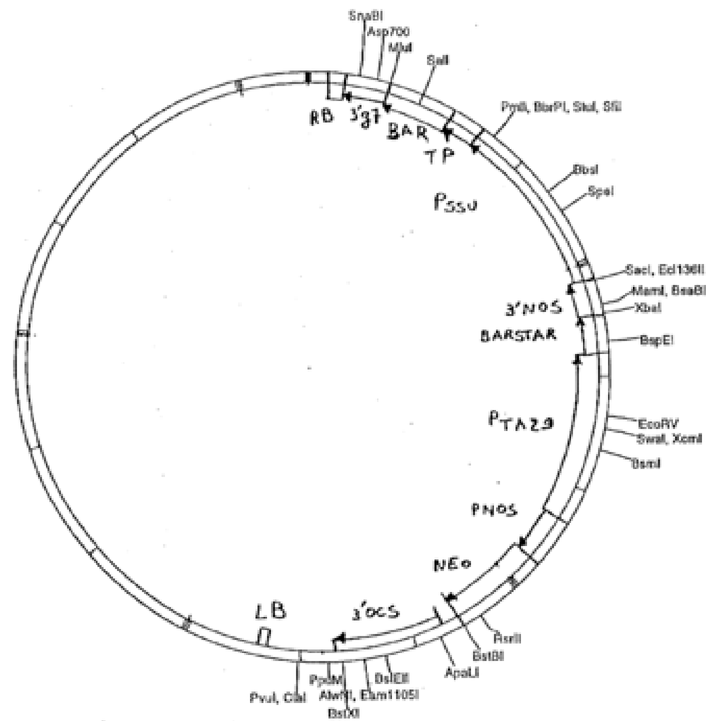

Figure 3-2 The restriction enzyme cleavage site of pTVE74RE

(Note: All the rights pertinent to the information in the diagram above and the responsibility for the content rest upon the applicant.)

(3) Method of preparing living modified organisms

1) Structure of the entire nucleic acid transferred in the recipient organism

In the MS1, *neo* gene expression cassette, *barnase* gene expression cassette and modified *bar* gene expression cassette (3'ocs-*neo*-Pnos-PTA29-*barnase*-3'nos-PSsuAra-modified *bar*-3'g7) in the T-DNA region were transferred which are located between LB and RB on the pTTM8RE (Figure 2-1). In addition, in the RF2, *neo* gene expression cassette, *barstar* gene expression cassette and modified *bar* gene expression cassette (3'ocs-*neo*-Pnos-PTA29-*barstar* -3'nos-PSsuAra-modified *bar*-3'g7) in the T-DNA region were transferred which are located between LB and RB on the pTVE74RE (Figure 3-1).

2) Method of transferring nucleic acid transferred to the recipient organism

The *Agrobacterium*-mediated transformation method was used for transferring nucleic acid to both MS1 and RF2 (Reference 18).

The *E.coli* MC1061 strain which possesses either pTTM8RE or pTVE74RE, the *E.coli* HB101 strain which possesses the transferable (helper) plasmid pRK2013, and the non-oncogenic *A.tumefaciens* C58C1Rif^R strain were coexistent. After the *A.tumefacines* C58C1Rif^R strain which possesses pTTM8RE or pTE74RE was produced, a piece of hypocotyls of the recipient organism was infected with it, and the T-DNA region between the RB and the LB was transferred into the oilseed rape genomes.

3) Processes of rearing of living modified organisms

(a) Mode of selecting the cells containing the transferred nucleic acid

After transformation, a piece of hypocotyls was grown in a solid medium containing 50 mg/L kanamycin-SO_4 or 20 mg/L phosphinothricin for selection. Then, the selected clones were moved to a hormone-free medium and regenerated to the plant body.

(b) Presence or absence of remaining *Agrobacterium* in case of using *Agro-bacterium* method for transferring nucleic acid

After transformation by the *Agrobacterium*, 500 mg/L of Carbenicillin was added to the medium and the remaining *Agrobacterium cells* were removed (Reference 18), therefore, the plant body of MS1 and RF2 contains no *Agrobacterium cell*. (Annex 3, Figure 2).

(c) Processes of rearing and pedigree trees of the following lines; cells to which the nucleic acid was transferred, the line with which the state of existence of replication products of transferred nucleic acid was confirmed, the line subjected to isolated field tests; and the line used for collection of other necessary information for assessment of Adverse Effect on Biological Diversity

After transformation, the regenerated plant body of each of MS1 and RF2 was comprehensively examined for the traits expressed and agricultural characteristics. The MS1RF2 was produced by crossing the line of BC2F1 or later generations of MS1 with the line of T3 or BC2F1 or later generations of RF2. The process of rearing of MS1RF2 is shown in Figure 4. The approvals of the MS1RF2 received from organizations in Japan are as follows.

[Food safety]

Based on the "Guideline for food safety assessment of food and food additives derived from recombinant-DNA technology", the conformity to the Guideline for safety of use as food regarding PGS2 and PHY36 in May 26, 1997 and regarding PHY23 in November 15, 1999 was confirmed by the Ministry of Health and Welfare (the Ministry of Health, Labour and Welfare, currently). In addition, along with legislating, passing through the "Procedures for food safety assessment of food and food additives derived from recombinant-DNA technology", safety of use for food was approved by the Ministry of Health, Labour and Welfare in March 30, 2001.

[Feed safety]

Based on the "Guideline for feed safety assessment of recombinant feed", the compatibility to the Guideline regarding glufosinate herbicide tolerant canola PGS2 and glufosinate herbicide tolerant canola PHY36 in June 13, 1997 and regarding glufosinate herbicide tolerant canola PHY23 in February 26, 1999 was confirmed by the Ministry of Agriculture, Forestry and Fisheries. In addition, along with legislating, passing through the "Procedures for feed safety assessment of feed and feed additives derived from recombinant-DNA technology", safety of use for feed was approved by the Ministry of Agriculture, Forestry and Fisheries in March 27, 2003.

[Environmental safety]

In 1996, based on the "Guideline for the use of recombinant in agriculture, forestry and fisheries," the compatibility to the guideline regarding the isolated field test on the recombinant oilseed rape was confirmed by the Ministry of Agriculture, Forestry and Fisheries. In addition, in April 4, 1997, the compatibility to the guideline regarding the recombinant oilseed rape being imported to Japan (used for processing and feed) was confirmed by the Ministry of Agriculture, Forestry and Fisheries.

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Figure 4 Pedigree tree of MS1RF2

(4) State of existence of nucleic acid transferred in cells and stability of expression of traits caused by the nucleic acid

1) Place where the replication product of transferred nucleic acid exists

It is expected that the genetic locus of both MS1 and RF2 (the original gene transformation) is heterozygote for the transferred gene locus. In addition, MS1, which is conferred the male sterile trait, is maintained by a cross with the non-recombinant oilseed rape and then, it is expected that a segregation ratio of 1:1 would be obtained in theory between glufosinate-tolerant and glufosinate-sensitive individuals under the control of single gene locus. Furthermore, it is expected that in the T1 generation raised by self-pollination of RF2 (the original gene transformation), a segregation ratio of 3:1 would be obtained in theory between glufosinate-tolerant and glufosinate-sensitive individuals and that in the population of the T2 generation strains obtained by self-fertilization among multiple individuals exhibiting the tolerance to glufosinate herbicide, a segregation ratio of 5:1 would be obtained in theory between glufosinate-tolerant and glufosinate-sensitive individuals.

As a result of examination on the segregation ratio between glufosinate-tolerant and glufosinate-sensitive individuals in various generations of MS1 and RF2, the BC1F1 and BC2F1 generations of MS1 exhibited the segregation ratio in good agreement with the theoretical segregation ratio (Annex 11, Table GBN114 and Table FBN9301[2]). In addition, the T1 generation of RF2 and the T2 generation obtained by self-pollination among multiple individuals exhibiting the tolerance to glufosinate herbicide showed the segregation ratio in good agreement with the theoretical segregation ratio (Annex 11, Table GBN030[1,3]). Consequently, as all the cases examined resulted in the segregation ratio as expected, the replication products of transferred nucleic acid in MS1 and RF2 are all considered to exist on the genome of oilseed rape at one site.

2) The number of copies of replication products of transferred nucleic acid and stability of its inheritance through multiple generations

In order to identify the number of copies of transferred nucleic acid, the genome DNA of MS1 (BC2F1 generation) and RF2 (T3 generation) was cleaved by the restriction enzymes, and the Southern blotting analysis was conducted using the modified *bar* probe, PSsuAra probe, PTA29 probe and *neo* probe. As a result, in each analysis, the band equivalent in size to the fragment derived from the vector was observed, and it was confirmed that one copy of T-DNA region was transferred in each of MS1 and RF2 (Annex 4-1, Figures 2a-d; Annex 4-2, Figures 2a-d). In addition, as a result of determination of nucleotide sequence (sequence analysis) in MS1 and RF2, it was confirmed that the identical sequence as in the T-DNA region on each plasmid was transferred (Annex 5-1 and Annex 5-2). The T-DNA region transferred in MS1 and RF2 is shown in Figure 5-1 and Figure 5-2 respectively.

Additionally, as the result of the Southern blotting analysis for the genome DNA of MS1 of the generations F1, BC2F1 and BC4F1 (obtained by the backcrossing with Cultivar A) and BC5F1 (obtained by the backcrossing with Cultivar B), 13kb band and 9kb band were detected in all generations as expected (Annex 4-1, Figure 3).

Moreover, as the result of the Southern blotting analysis for the genome DNA of RF2 of the generations T1, T3 and BC2F1 (obtained by the backcrossing with Cultivar C), 14kb band and 7kb band were detected in all generations as expected (Annex 4-2, Figure 2). As a result, it was confirmed that the transferred genes into MS1 and RF2 are inherited stably through the multiple generations.

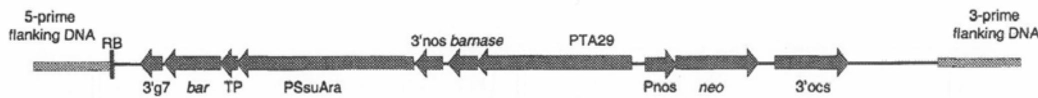

Figure 5-1 Map of the entire T-DNA region transferred to MS1

In the diagram, *bar* refers to the modified *bar* gene and TP refers to the transport peptide.

(Note: All the rights pertinent to the information in the diagram above and the responsibility for the content rest upon the applicant.)

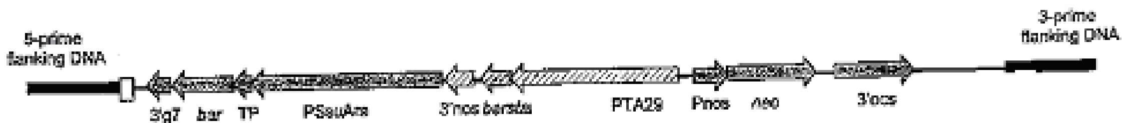

Figure 5-2 Map of the entire T-DNA region transferred to RF2

In the diagram, *bar* refers to the modified *bar* gene and TP refers to the transport peptide.

(Note: All the rights pertinent to the information in the diagram above and the responsibility for the content rest upon the applicant.)

3) The position relationship in the case of multiple copies existing in chromosome

As mentioned above, MS1RF2 contains one copy of modified *bar* gene on the chromosome derived from the parent plants and then, possesses two copies of modified *bar* gene.

4) Inter-individual or inter-generational expression stability under a natural environment with respect to the characteristics referred to specifically in (6)-1)

[Modified *bar* gene]

The expression of the modified *bar* gene was confirmed by the glufosinate herbicide spraying test. Since the genetic locus of MS1 is heterozygote for the tolerance to glufosinate herbicide, about 50% of MS1 individuals are expected in theory to exhibit the tolerance to glufosinate herbicide, while regarding RF2 and MS1RF2, 100% of individuals are expected to exhibit glufosinate tolerance.

As a result of field tests conducted in foreign countries at several areas on the segregation ratio between glufosinate-tolerant and glufosinate-sensitive individuals, about 50% of MS1 individuals of the BC1F1 generation and BC3F1 generation (obtained by the backcrossing with Cultivar A) (Annex 11, Table GBN114, Table $FNB147_1$), and 100% of RF2 individuals of the T3 generation and T5 generation exhibited the tolerance to glufosinate herbicide (Annex 11, Table $FNB9301_2$, Table $FNB9401_2$), and also in the MS1RF2 (BC2F1 generation of MS1×T4 generation of RF2) about 100% of individuals showed the glufosinate tolerance (Annex 11, Table $FBN9401_2$). Consequently, since individuals in all generations showed the segregation ratio as expected, it was confirmed that the modified *bar* gene is stably expressed across the individuals and multiple generations.

[*barnase* gene and *barstar* gene]

The expression of the *barnase* gene in MS1 and the *barstar* gene in RF2 and MS1RF2 was confirmed by the expression of individuals exhibiting male fertility and male sterility. MS1 is maintained by a crossing with the non-recombinant oilseed rape and then, it is expected that MS1 would contain male-sterile individuals caused by expression of the *barnase* gene and male-fertile individuals at a segregation ratio of 1:1 in theory. On the other hand, it is expected that for RF2, all the individuals would exhibit male fertility and for MS1RF2, all the individuals would exhibit male fertility due to expression of the BARSTAR protein which inhibits the ribo-nuclease activity of BARNASE protein.

In the test conducted in foreign countries at several areas, for MS1 (BC2F1 generation), about 50% of individuals exhibited male fertility (Annex 11, Table $FBN9302_6$). RF2 (T3 generation and T5 generation) and MS1RF2 [MS1(BC2F1) × RF2(T3) and MS1(BC2F1) × RF2(T4)], about 100% of individuals exhibited male fertility (Annex 11, Table $FNB9301_6$, Table $FNB9401_5$). Consequently, since all individuals in all generations almost showed the segregation ratio as expected, it is considered that the *barnase* gene and the *barstar* gene are stably inherited under a natural environment.

Moreover, in order to examine the expression of the modified *bar* gene, *barnase* gene (only for MS1), *barstar* gene (only for RF2) and *neo* gene transferred in the MS1 and RF2, Northern blotting analysis was conducted for the leaves, flower buds, seeds and pollens of the MS1 (BC2F1 generation) and RF2 (T3 generation).

[MS1]

The transcripts of the modified *bar* gene were detected only in leaves (detection limit: 0.1 pg/µg total RNAs). In addition, the transcripts of the *barnase* gene were not detected in any tissue (detection limit: 0.4 pg/µg total RNAs). Furthermore, the transcripts of the *neo* gene were not detected in any tissue (detection limit: 0.2 pg/µg total RNAs) (Annex 6, Table 2).

[RF2]

The transcripts of the modified *bar* gene were detected in leaves, floral stem and flower buds, but not detected in any other tissues (detection limit: 0.2 pg/µg total RNAs). In

addition, the transcripts of the *barstar* gene were detected only in flower buds (detection limit: 0.1 pg/µg total RNAs). Moreover, the transcripts of the *neo* gene were not detected in any tissue (detection limit: 0.1 pg/µg total RNAs) (Annex 6, Table 1).

In addition, regarding to the enzyme activity of the modified PAT protein in leaves of MS1 in the individual generations of F1, BC2F1, BC4F1 (obtained by the backcrossing with Cultivar A) and BC4F1 (obtained by the backcrossing with Cultivar B) and RF2 in the generations T1, T3, BC2F1 (obtained by the backcrossing with Cultivar C), all generations were examined by the spectrophotometric method. As a result, enzyme activity was confirmed in all the generations of both lines examined (Annex 7, Table L12, Table L11). Moreover, as a result of measurements for enzyme activity of the NPT II protein in those generations of MS1 and RF2, the activity of the NPT II protein was confirmed in all the generations of both lines examined (Annex 8, Figure $L11_1$; Figure $L10_1$).

5) Presence or absence, and if present, degree of transmission of nucleic acid transferred through virus infection and/or other routes to wild animals and wild plants

   MS1 and RF2 contain no DNA sequence which possesses transferring factor and therefore, there is no possibility of transmission of nucleic acid transferred to wild animals and wild plants under a natural environment.

(5) Methods of detection and identification of living modified organisms and their sensitivity and reliability

Detection of MS1RF2 is available by PCR method using the flanking sequences of DNA transferred in MS1 and RF2 respectively for each plant body (seed). This PCR method is utilized effectively for cultivation management of individual events (Annex 12).

(6) Difference from the recipient organism or the taxonomic species to which the recipient organism belongs

1) Details of properties conferred as a result of the expression of copies of the transferred nucleic acid

   MS1 is given the traits of tolerance to glufosinate herbicide and male sterility, and RF2 is given the traits of tolerance to glufosinate herbicide and fertility restoration. Crossing between MS1 and RF2 allows restoration of fertility in the F1 generation since the BARNASE protein forms non-covalently bonded complex specifically with BARSTAR protein in one-to-one correspondence in the anther tapetum cells and inhibits the activity of BARNASE protein.

2) With respect to the physiological or ecological characteristics listed below, presence or absence of difference between modified plant and the taxonomic species to which the recipient organism belongs, and the degree of difference, if present

In 1996, the isolated filed tests were conducted at the Hokkaido Agricultural Research Center, the comparison was made between MS1RF2 {[MS1(obtained by the backcrossing with Cultivar X)×RF2 (three or more self-pollination posterity of T0)], corresponding to the "PGS2" in Annex 9}, and the control type oilseed rape (produced by the crossing with Cultivar X and Drakkar, hereinafter referred to as "the non-recombinant oilseed rape", corresponding to the "non-recombinant PGS2" in Annex 9), and the differences between them were examined (Annex 9). Moreover, in 2006, in the special screened greenhouse in Japan, examination was made regarding the heat-tolerance at the early stage of growth, fertility and size of the pollen, dormancy of the seed, and productivity of harmful substances to make comparison between MS1RF2［BC6F1 generation of the MS1×T6 generation of the RF2］ and the recipient cultivar, Drakkar (Annex 10).

For references of evaluation, results of cultivation tests conducted in foreign countries (Annex 11) at several areas were used.

(a) Morphological and growth characteristics

In isolated fields, comparison was made between MS1RF2 and the non-recombinant oilseed rape for the plant height, the number of primary branches, dry weight of aerial parts (stems and leaves), plant shape, color of leaves, time of bolting, flowering period, maturation period, rate of pods formation, length of pod, the number of seed setting (seeds/pod) and color of seed. As a result, MS1RF2 showed slightly lower value in plant height and weight of aerial parts (stems and leaves) compared to the non-recombinant oilseed rape. In other items examined, there was no significant difference between MS1RF2 and the non-recombinant oilseed rape (Annex 9, Tables 1 and 2).

(b) Cold-tolerance or heat-tolerance at the early stage of growth

Seedlings of MS1RF2 and Drakkar were raised under the conditions (35℃ and 12-hours day length and 12-hours night length) and as a result, it was confirmed at one month later observation that all the individuals had died (Annex 10, Table 23). Therefore, it is considered that the heat-tolerance of MS1RF2 at the early stage of growth is as low as that of Drakkar.

The oilseed rape varieties sown in autumn in Japan are generally known to grow even in winter in both warm and cold districts, though the rate of growth varies (Reference 82).

(c) Wintering ability or summer survival of the matured plant

As a result of observation for the summer survival of MS1RF2 in the isolated fields, it was observed as being equal as that of the non-recombinant oilseed rape and the other varieties examined (Annex 9).

It is generally known that oilseed rape shows high cold tolerance and high snow endurance (Reference 82).

(d) Fertility and size of the pollen

Pollens were collected from the MS1RF2 and Drakkar cultivated in the special screened greenhouse, and stained with acetocarmine solution and observed under a microscope. As a result, 99% of the pollens from the both lines were found stained, showing a high fertility of the pollens (Annex 10). In addition, as a result of comparison of size of pollen, no statistically significant difference was observed (Annex 10, Table 21).

(e) Production, shedding habit, dormancy, and germination rate of the seed

In isolated field tests, the seed yield per plant (g/plant) of MS1RF2 showed 4.8 g heavier than that of the non-recombinant oilseed rape. MS1RF2 showed no significant difference in the 1000-seed weight from the non-recombinant oilseed rape (Annex 9, Table 2).

In cultivation tests conducted in foreign countries, the seed yield (kg/ha) of MS1RF2 was compared to that of Drakkar in seven areas in 1993 and five areas in 1994. As a result, in all years and areas, there was no statistically significant difference between MS1RF2 and Drakkar (Annex 11, Table $FBN9301_8$, Table $FNB9401_8$).

Regarding the shedding habit, comparison was made for the rate of open pods formed in isolated field tests and as a result, MS1RF2 and the non-recombinant oilseed rape were both found easy to open pods similarly to each other without any difference (Annex 9, Table 2).

To evaluate the germination rate, 20 seeds each harvested from MS1RF2 and Drakkar cultivated in the special screened greenhouse were sown. As a result, one week after sowing, the germination rate was found 95.0% (19/20 grains) for the MS1RF2 and 85.0% (17/20 grains) for Drakkar (Annex 10, Table 24). In addition, the seeds that were found not germinated were evaluated for life or dead based on the tetrazolium method, and as a result, all seeds decayed and inside of the seed coat were lost, and the seeds were not clearly stained. It was confirmed that the cause of non-germination of the seeds is death, not dormancy. Based on the understanding that the seeds found surviving one week after sowing all germinated successfully, the MS1RF2 and Drakkar are considered to possess extremely low dormancy.

(f) Crossability

No test was conducted for the crossability of MS1RF2. However, the fertility of pollens of MS1RF2 examined in the special screened greenhouse in 2006 was equivalent as that of Drakkar (Annex 10), and it was confirmed that there was no statistically significant difference between them for the size of pollens (Annex 10, Table 21), and there was no statistically significant difference between MS1RF2 and the recipient organisms for the seed yield (kg/ha) compared in 1993 and 1994 in foreign countries at several areas (Annex 11, Table $FBN9301_8$; Table $FNB9401_8$). Therefore, it is expected that the crossability of MS1RF2 would be almost equal as that of Drakkar.

(g) Productivity of harmful substances

In order to check whether the substances are excreted from the roots of MS1RF2 which can affect other plants, exists in the plant body which can affect other plants after dying, and are excreted from the roots which can affect microorganisms in soil, the succeeding crop test, plow-in test and soil microflora test were carried out respectively in the special screened greenhouse.

Succeeding crop test： After cultivating the recombinant oilseed rape MS1RF2 and Drakkar for about two months, radishes were cultivated as test plants in the remaining soil respectively, and the comparison was made for germination rate, plant height, root length, fresh weight and dry weight of radishes. As a result, in all the items examined, no statistically significant difference was observed (Annex 10, Tables 1, 3, 4, 6 and 7). Therefore, it is considered that the recombinant oilseed rape MS1RF2 has not acquired any productivity of the substances excreted from the roots which can affect other plants.

Plow-in test：The dried powder of plant body of the MS1RF2 and Drakkar cultivated about three months after sowing was mixed with soil (1%), respectively, and seeds of radish were sowed in the soil and cultivated. Then the comparison was made for germination rate, plant height, root length, fresh weight and dry weight. As a result, there was no statistically significant difference observed in any items (Annex 10, Tables 9, 11, 13, 14, 16, and 17). Therefore, it is considered that MS1RF2 has not newly acquired any productivity of the substances which can affect other plants after dying.

Soil microflora test：The soil was obtained after cultivating the recombinant oilseed rape MS1RF2 and Drakkar for about two months, and was diluted by adding sterilized phosphate buffer solution. bacteria and actinomyces were incubated in PTYG medium, and filamentous fungi were incubated in Rose Bengal medium, and the comparison was made for the number of each microorganisms. As a result, there was a statistically significant difference observed in the number of bacteria and actinomyces between MS1RF2 and Drakkar, and MS1RF2 exhibited higher values than Drakkar (Annex 10, Table 19). Based on the above results, it is considered that MS1RF2 has not newly acquired any productivity of the substances excreted from the roots which can affect the decrease in viable cell count in microorganisms in soil.

II. Review by persons with specialized knowledge and experience concerning Adverse Effect on Biological Diversity

A review was made by persons with specialized knowledge and experience concerning Adverse Effect on Biological Diversity (called Experts) for possible Adverse Effect on Biological Diversity caused by the use in accordance with the Type 1 Use Regulation for Living Modified Organism based on the "Law concerning the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms."

Results of the review are listed below.

(Note 1) The underlined description in the following sections is intended to alert the readers to differences between three varieties of stack line oilseed rape.

MS1RF2 is produced by a cross between the glufosinate herbicide tolerant and male sterile oilseed rape (MS1) and the glufosinate herbicide tolerant and fertility restored oilseed rape (RF2). MS1RF2 produces the modified PAT protein derived from the both parent plants, the BARNASE protein derived from MS1, and the BARSTAR protein derived from RF2.

In the MS1RF2, the BARNASE protein and the BARSTAR protein interact with each other in the anther tapetum cell [Note 2] (non-covalently bonding specifically to each other in one-to-one correspondence) and as a result, the ribo-nuclease activity of BARNASE protein is inhibited by the BARSTAR protein and the male fertility is restored. As mentioned above, MS1RF2 exhibits the interaction of the traits derived from the both parent lines and then, evaluation has been focused on the stack line oilseed rape rather than the both parent lines.

The modified PAT protein possesses high substrate specificity and thus, it is considered not to interact with the BARNASE protein and the BARSTAR protein.

(Note 2) By a cross between the female strain (male-sterile MS1) and the male strain (fertility restored RF2), seeds of the first cross cultivar (F1) is obtained which possesses the male fertility.

1. Item-by-item assessment of Adverse Effect on Biological Diversity

(1) Competitiveness

Oilseed rape (*Brassica napus* L.) to which the recipient organism belongs was introduced to Japan in early Meiji period, and it is reportedly growing on river banks, along roadsides, in the surroundings of seed off-loading harbors, and in other such areas. It is generally known that oilseed rape would be eventually replaced with perennial plants and shrub in the environmental conditions without any regular disturbance such as roadsides, cliffs and riverside areas.

In the isolated fields and special screened greenhouses in Japan, a total of 19 traits relating to the competitiveness of MS1RF2 were examined based on the comparison with the control plants.

As a result, for the plant height and the weight of stems and leaves, MS1RF2 showed the slightly lower values. For the seed yield per plant, MS1RF2 showed the relatively higher value. However, these differences are considered not to cause MS1RF2 to become competitive.

MS1RF2 is given;

(i) a trait to be tolerant to glufosinate herbicide, though it is generally considered that the glufosinate does not exert selective pressure under a natural environment.

(ii) MS1RF2 produces pollens due to the interaction between the BARNASE protein derived from MS1 and the BARSTAR protein derived from RF2, though the pollens of MS1RF2 are found to have equivalent fertility as those of Drakkar, and for the size of the pollens, it is confirmed that there is no significant difference from the control plants.

(iii) The BARNASE protein degrades RNA in the tapetum cells of the recipient organism, though there is no report that it possesses activity against any other substrates.

(iv) There is no report that the BARSTAR protein possesses any function other than that which inhibits the ribo-nuclease of the BARNASE protein.

Therefore, it is considered unlikely that these traits could cause MS1RF2 to become competitive under a natural environment.

Based on the above understanding, it was judged that the conclusion by the applicant that the wild animals and wild plants likely to be affected cannot be specified and that the use of this recombinant oilseed rape poses no significant risk of Adverse Effect on Biological Diversity attributable to competitiveness is reasonable.

(2) Productivity of harmful substances

It has been confirmed that the contents of erucic acid and glucosinolate in the MS1RF2, which are recognized as harmful substances to human and other mammals, fall within the ranges for the cultivars known as canola, in which the erucic acid and glucosinolate content was reduced by selective breeding.

MS1RF2 produces the modified PAT protein derived from the both parent plants, the BARNASE protein derived from MS1, and the BARSTAR protein derived from RF2. The modified PAT protein possesses high substrate specificity and then, it is considered unlikely to transfer the acetyl group to any substances other than the substrate glufosinate. In addition, in the MS1RF2, the BARNASE protein and the BARSTAR protein non-covalently bind specifically to each other in the anther tapetum cells in one-to-one correspondence, and the ribo-nuclease activity of BARNASE protein is inhibited by the BARSTAR protein. Therefore, it is considered unlikely that these proteins affect any other metabolic systems in the plant body and newly produce any harmful substances.

In addition, for the amino acid sequences of those proteins, comprehensive homology search and allergen epitope homology search were conducted. Consequently, no homology with any known toxin and allergen was observed.

In the special screened greenhouses in Japan, the succeeding crop test, soil microflora test and plow-in test have been conducted to check the harmful substances productivity of this recombinant oilseed rape (the substances excreted from the roots which can affect other plants, the substances excreted from the roots which can affect microorganisms in soil, and the substances existing in the plant body which can affect other plants after dying).

In the succeeding crop test and plow-in test, no statistically significant difference was observed between MS1RF2 and Drakkar. On the other hand, for the number of bacteria, actinomyces and filamentous fungi examined in the soil microflora test, a statistically significant difference was observed in the number of bacteria and actinomycete, though MS1RF2 showed higher values compared to Drakkar and then, it is considered that MS1RF2 has not newly acquired any productivity of the substances excreted from the roots which can affect the decrease in viable cell count in microorganisms in soil.

Based on the above understanding, it was judged that the conclusion by the applicant that the wild animals and wild plants likely to be affected cannot be specified and that the use of this recombinant oilseed rape poses no significant risk of Adverse Effect on Biological Diversity attributable to productivity of harmful substances is reasonable.

(3) Crossability

In a natural environment in Japan, a number of plants of the family *Brassicaceae* are growing, though known species that can be crossed with oilseed rape (*Brassica napus* L.) include *B. rapa* L. (turnip, Komatsuna, conventional rapeseed, etc.) of the genus *Brassica*; *B. juncea* (L.) Czern (mustard, leaf mustard, etc.); *B. nigra* (L.) W.D.J.Koch (black mustard) and *Raphanus raphanistrum* L. (wild radish) in addition to oilseed rape itself.

Oilseed rape, *B. juncea*, *B. nigra*, and *R. raphanistrum* are regarded as all introduced species brought into Japan artificially after Meiji period. In addition, *B. rapa* is also a cultivar-derived introduced species though it was introduced to Japan in olden times. As such, these are not specified as wild species as to be possibly affected.

Based on the above understanding, it was judged that the conclusion by the applicant that the wild animals and wild plants likely to be affected cannot be specified and that the use of this recombinant oilseed rape poses no significant risk of Adverse Effect on Biological Diversity attributable to crossability is reasonable.

(4) Additional information

The possible indirect Adverse Effect on Biological Diversity attributable to crossing of recombinant oilseed rape with non-recombinant oilseed rape and the related species described on the above was evaluated. The possible indirect Adverse Effect on Biological Diversity refers to that; i) hybrid produced by crossing would become competitive and exterminate species population of the other wild animals and wild plants, and ii) related species population would decrease due to the effect of transferred gene spread by crossing, and wild animals and wild plants such as insects which are dependent on the related species would be affected for maintenance of their population.

In concrete,

(i) In the comparison between MS1RF2 and Drakker, there was no difference for the fertility of pollens, and no statistically significant difference was found in the size of pollens and the seed yield per plant. Consequently, it is expected that there is no difference for the crossability between them.

(ii) Regarding the crossability with related species, it is reported that it would be hard to produce hybrid, and even if hybrid is produced, the progeny would possess low fertility.

(iii) It is considered unlikely that MS1RF2 which possesses glufosinate tolerance and fertility restoration would become competitive under a natural environment.

(iv) It is reported that the cross-progeny of plants which possess male sterility as dominant trait would decrease its population rapidly over generations.

Consequently, it is judged that the possibility that MS1RF2 would cross with related species to produce hybrid and the progeny would become competitive under a natural environment is as low as oilseed rape to which the recipient organism belongs.

In addition,

(v) In the comparison between the individuals tolerant and not tolerant to glufosinate herbicide in the BC3 generation obtained by three-time repeated backcrossing of *B. rapa* with the hybrid between the recombinant oilseed rape, which contains both the modified *bar* gene and the *barstar* gene, and the *B. rapa*, through screening with glufosinate herbicide, it was reported that there was no difference in the fertility of pollen, survivability and the amount of seeds produced.

(vi) Even if the *barnase* gene become out of control of the promoter PTA29 and acquire the promoter which would express constitutively or site-specifically in plant, the possibility that plant would grow properly is low, and it is considered unlikely that the gene would spread extensively in the related species population.

Consequently, it is considered that the possibility that the genes would affect maintenance of interspecies hybrid population in a shorter period of time is low.

Based on the above understanding, it was judged that the conclusion by the applicant that there is no risk of indirect Adverse Effect on Biological Diversity attributable to crossability is reasonable.

2. Conclusion based on the Biological Diversity Risk Assessment Report

Based on the above understanding, the Biological Diversity Risk Assessment Report concluded that there is no risk that the use of this stack line recombinant oilseed rape (MS1RF2) in accordance with Type 1 Use Regulation causes Adverse Effect on Biological Diversity. It was judged that the conclusion above made by the applicant is reasonable.

Reference

Confidential: Not made available or disclosed to unauthorized person

Reference material

Reference material 1：Outline of the Biological Diversity Risk Assessment Report (MS1)
Reference material 2：Outline of the Biological Diversity Risk Assessment Report（RF2）

Annex

Annex	1-1：	Entire nucleotide sequence of plasmid pTTM8RE Confidential: Not made available or disclosed to unauthorized person
	1-2：	Entire nucleotide sequence of plasmid pTVE74RE Confidential: Not made available or disclosed to unauthorized person
Annex	2：	Confirmation of sequences outside of T-DNA region in MS1 and RF2 Confidential: Not made available or disclosed to unauthorized person
Annex	3：	Presence or absence of remaining *Agrobacterium* in MS1 and RF2 Confidential: Not made available or disclosed to unauthorized person
Annex	4-1：	Molecular analysis in MS1 Confidential: Not made available or disclosed to unauthorized person
	4-2：	Molecular analysis in RF2 Confidential: Not made available or disclosed to unauthorized person
Annex	5-1：	Sequence analysis on the transferred genes in the male sterile oilseed rape MS1 Confidential: Not made available or disclosed to unauthorized person
	5-2：	Sequence analysis on the transferred genes in the fertility restored oilseed rape RF2 Confidential: Not made available or disclosed to unauthorized person
Annex	6：	Result of Northern blotting analysis in MS1 and RF2 Confidential: Not made available or disclosed to unauthorized person
Annex	7：	PAT activity in MS1 and RF2 Confidential: Not made available or disclosed to unauthorized person
Annex	8：	NPT II activity in MS1 and RF2 Confidential: Not made available or disclosed to unauthorized person
Annex	9：	Isolated field test report Confidential: Not made available or disclosed to unauthorized person
Annex	10：	Report on the tests in the special screened greenhouse Confidential: Not made available or disclosed to unauthorized person
Annex	11：	Report on cultivation tests carried out in foreign countries Confidential: Not made available or disclosed to unauthorized person
Annex	12：	Event Identifying Method for MS1 and RF2 Confidential: Not made available or disclosed to unauthorized person